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Molecular epidemiology, genome characterization, and recombination event of human parechovirus

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ABSTRACT

Human Parechovirus (HPeV), a member of the *Picornaviridae* family, is an infectious agent mostly affecting children. There are 16 recognized genotypes which have globally spread. This study incorporated a total of 2957 nasopharyngeal (NP) swab and 759 fecal samples that were collected from different parts of Thailand. The NP of HPeV was detected in 0.4% of NP swab and 6.1% of fecal samples. The majority of HPeV infections occur in infants below the age of 2 years, while infections were detected in children above the age of 10 years as well. Various genotypes comprising 1A, 1B, 2, 3, 4, 5, 6, 10 and 14 have been characterized. This study revealed recombination events in 16 samples in which HPeV1B was shown as the highest frequency. In conclusion, HPeV can be detected in both the respiratory and GI tract. Moreover, HPeV which circulates in Thailand is highly diverse and subject to recombination.

Introduction

Human parechovirus (HPeV) is a small non-enveloped single stranded RNA virus of positive polarity which belongs to the vast family, *Picornaviridae*. The virus has first been discovered from an outbreak of diarrhea among children in 1961. Based on their serology and clinical presentation, the virus was defined as echovirus 22 and 23 of the genus *Enterovirus* (Wigand and Sabin, 1961). However, studies on genetics, protein translation and biological properties of the virus have shown that it is different from other members of the genus *Enterovirus*. Hence, it has been reclassified into a new genus, *Parechovirus* and those viruses previously defined as echovirus 22 and 23 were re-named HPeV1 and HPeV2, respectively (Stanway and Hyypia, 1999; Stanway et al., 1994). Recently, additional types of HPeV have been reported, which were associated with different clinical manifestations. For example, HPeV3 was isolated from nasopharyngeal aspirates (NPA) and related to sepsis in neonates (Ito et al., 2004). HPeV4 was isolated from fecal samples and related to fever in neonates (Benschop et al., 2006a). HPeV5 had previously been defined as HPeV2 based on serology of children presenting with high fever but upon genome analysis was re-classified as type five (Oberste et al., 1998). HPeV6 was isolated from one child suffering from Reye's syndrome (Watanabe et al., 2007), HPeV7 was identified by metagenomic method in fecal samples of a healthy child (Li et al., 2009), HPeV8 was isolated from fecal samples during an outbreak of acute diarrhea in Brazil (Drexler et al., 2009), and HPeV9 to HPeV16 which unpublished but had already been assigned by the following website (http://www.picornastudygroup.com/types/parechovirus/hpev.html).

Since the first report of HPeV infection, the epidemics of virus have been continuously reported. The most common genotype of the virus that could be isolated worldwide was HPeV1B followed by HPeV3. In contrast, other genotypes such as HPeV1A, HPeV2, and HPeV4-6 were less common among infected patients than those two dominant genotypes (Benschop et al., 2008b; Ito et al., 2010; Pham et al., 2010; Tapia et al., 2008). It should be noted that HPeV7 to 16 are a new genotypes that have been recently discovered. Thus, epidemiology and prevalence of these genotypes have not been fully established. The virus was mostly detected in children especially in infants below the age of 3 years (Benschop et al., 2006b; Tauriainen et al., 2007; Verboon-Maciolek et al., 2005). However, patients above the age of 10 years have also been noted (Abed and Boivin, 2006; Figueroa et al., 1989; Tapia et al., 2008; Watanabe et al., 2007). The longitudinal surveillance in USA between 1983 and 2005 has shown 3% of HPeV1 and 68% of HPeV2 had been isolated from infants less than 1 year old (Khetsuriani et al., 2006). In contrast, a study conducted in the Netherlands in 2000 has proposed that children below the age of 3 years were infected with HPeV1 and HPeV3 (Benschop et al., 2006b). The clinical presentations of HPeV infection were associated with mild disease of the respiratory and gastrointestinal (GI) tract (Harvala et al., 2008; Zhang et al., 2010; Zhong et al., 2011). According to a recent study, HPeV, especially HPeV3, was strongly associated with sepsis in neonates (Boivin et al., 2005; Harvala et al., 2009).

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The HPeV genome comprises approximately 7300 nucleotides flanked by an un-translated region (UTR) at both the 5′ and 3′ end. The virus translates a polyprotein with one single open reading frame (ORF) which is subsequently cleaved during the post-translational process into 3 structural proteins, VP0, VP3 and VP1, and 7 non-structural proteins, 2A–2C and 3A–3D (Harvala and Simmonds, 2009; Harvala et al., 2010).

Based on differences in antigenicity along with vast diversities in the virus genome, 16 types of HPeV have been classified (http://www.picornastudygroup.com/types/parechovirus/hpev.html). Rapid changes in their genomes normally occurring in the course of the replication cycle and thus contributing to antigenic drift with substitution rates ranging from 2.2×10^{-3} to 2.8×10^{-3} have been shown (Faria et al., 2009). Like with other picornaviruses, recombination in their genome had been detected and the breaking point of recombination was shown at the junction between P1 and P2 and the 5′UTR and P1 region (Benschop et al., 2008a, 2010; Drexler et al., 2011; Williams et al., 2009; Zoll et al., 2009). The different clusters on the phylogenetic tree of structural and non-structural genes have been clearly shown in a previous study (Calvert et al., 2010) which pointed out that recombination had occurred; however, both process and effect of this event in HPeV have remained unclear.

Table 1
Summary of HPeV positive samples.

Samples code	Samples type	Age	Sex	Other virus	HPeV type	3Dpol clade
CU-B402	Fecal	6 Mo	F	Negative	1B	J
CU-B408	Fecal	3 Mo	M	Positive Rota	1B	J
CU-B433	Fecal	1 Yr	F	Negative	1B	BA
CU-B463	Fecal	5 Mo	M	Positive Rota	1B	Z
CU-B478	Fecal	6 Mo	F	Negative	1B	O′*
CU-B486	Fecal	11 Mo	F	Negative	5	BA
CU-B500	Fecal	12 Yr	M	Negative	1B	O′*
CU-B579	Fecal	2 Mo	F	Positive Rota	1B	J
CU-B580	Fecal	9 Mo	F	Negative	1B	J
CU-B587	Fecal	11 Mo	M	Negative	1B	J
CU-B588	Fecal	3 Mo	F	Negative	1B	J
CU-B595	Fecal	10 Mo	F	Negative	1B	J
CU-B596	Fecal	11 Mo	M	Negative	1B	J
CU-B606	Fecal	NS	NS	Positive Rota	5	AE′*
CU-B615	Fecal	NS	NS	Positive Rota	1B	AE′*
CU-B628	Fecal	1 Yr	F	Negative	1B	BA
CU-B636	Fecal	1 Yr	M	Negative	1B	O′*
CU-B685	Fecal	1 Yr	F	Negative	3	W
CU-B688	Fecal	3 Mo	M	Negative	1B	BA
CU-B705	Fecal	6 Mo	M	Negative	4	Y
CU-B711	Fecal	1 Yr	M	Positive Rota	1A	K′*
CU-B806	Fecal	10 Mo	F	Negative	1B	BA
CU-B811	Fecal	7 Mo	M	Positive Rota	1B	J
CU-B812	Fecal	11 Mo	M	Positive Rota	6	BD
CU-B825	Fecal	6 Mo	F	Positive Rota	1A	BA
CU-B846	Fecal	1 Yr	M	Negative	1B	W
CU-B847	Fecal	1 Yr	F	Negative	2	BC′*
CU-B850	Fecal	5 Mo	M	Negative	2	BC′*
CU-B851	Fecal	6 Mo	M	Negative	1B	BA
CU-B852	Fecal	5 Mo	M	Negative	2	BC′*
CU-B865	Fecal	5 Mo	M	Negative	2	BC′*
CU-B867	Fecal	1 Yr	F	Negative	2	BC′*
CU-B868	Fecal	9 Mo	M	Negative	1B	J
CU-B869	Fecal	10 Mo	M	Negative	6	BD
CU-B874	Fecal	1 Yr	F	Negative	6	BD
CU-B903	Fecal	6 Mo	M	Negative	1B	G′*
CU-B961	Fecal	6 Mo	M	Positive Rota	14	BD
CU-B984	Fecal	1 Yr	M	Negative	6	AB′*
CU-B1002	Fecal	1 Yr	M	Positive Rota	1B	BA
CU-B1009	Fecal	10 Mo	F	Positive Rota	6	BD
CU-B1046	Fecal	1 Yr	M	Positive Rota	6	BD
CU-B1050	Fecal	1 Yr	M	Positive Rota	6	BD
CU-B1055	Fecal	9 Mo	M	Positive Rota	6	BD
CU-B1064	Fecal	10 Mo	M	Positive Rota	6	BD
CU-B1091	Fecal	8 Mo	M	Negative	14	AB′*
CU-B1154	Fecal	16 Yr	F	Negative	1B	J
CU-H403	NP sawab	NS	F	Negative	1B	O′*
CU-H2107	NP sawab	NS	F	Positive H3	1A	AN
CU-H2108	NP sawab	NS	M	Negative	4	AN
CU-H2159	NP sawab	NS	F	Negative	6	BD
CU-H2779	NP sawab	49 Yr	F	Negative	6	BD
CU-C315	NP sawab	9 Mo	F	Negative	1B	Z
CU-C534	NP sawab	2 Yr	F	Negative	6	BD
CU-C561	NP sawab	2 Yr	F	Negative	1B	Z
CU-C724	NP sawab	7 Yr	F	Negative	10	K′*
CU-C763	NP sawab	2 Yr	M	Negative	6	BD
CU-C936	NP sawab	1 Yr	M	Negative	6	BD
CU-C1204	NP sawab	3 Yr	F	Negative	6	BD

Abbreviation: Mo; Month, Yr; Year, NS; Not specify.
* The new 3Dpol clade defined in this study.

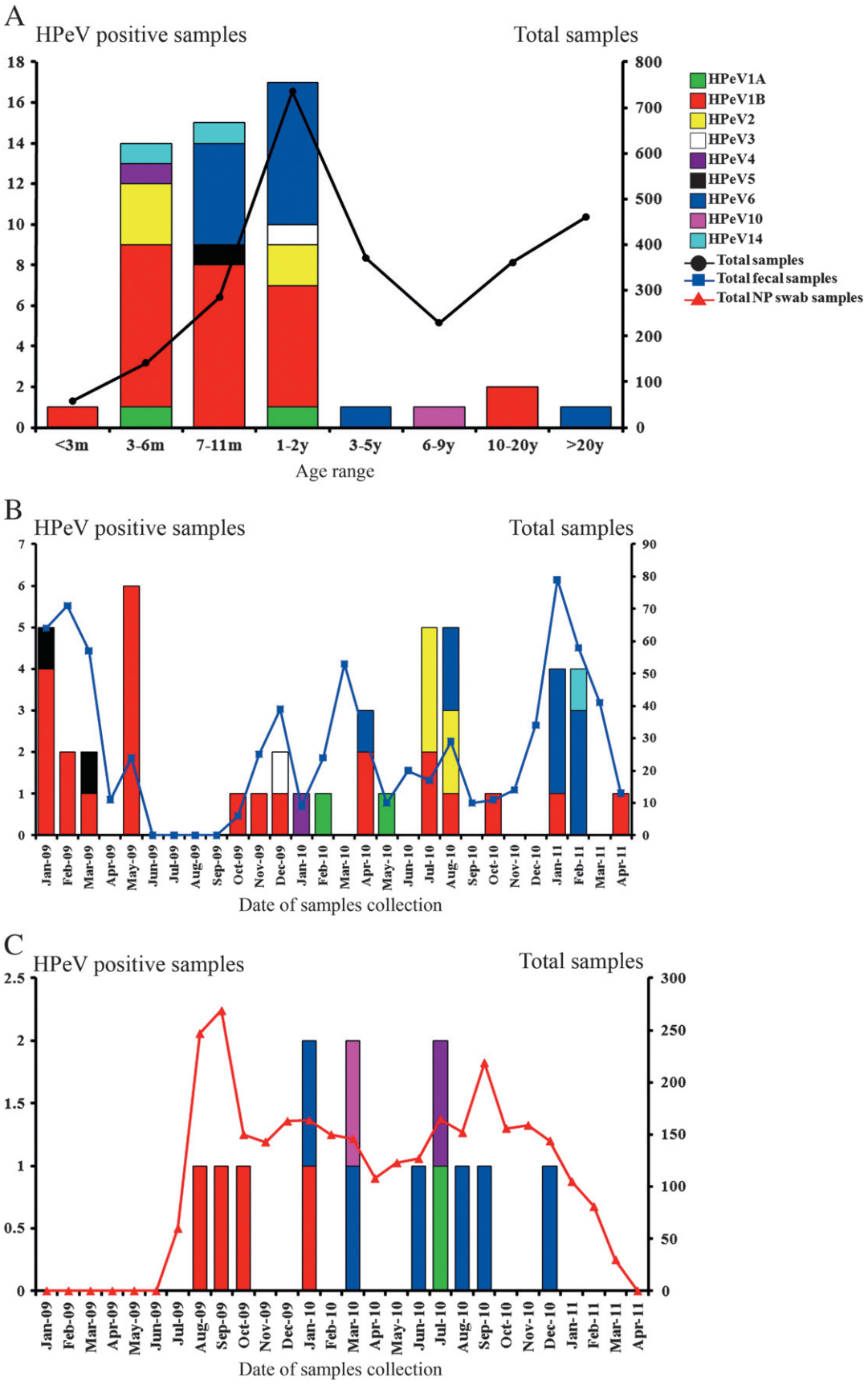


Fig. 1. Age distribution (A) and collection period of fecal (B) and NP swab (C) samples. The positive HPeV samples are shown as relative percentage of all samples collected.

In this study, we investigated the molecular epidemiology of HPeV in respiratory and fecal samples collected in different parts of Thailand from Jan 2009–Jan 2011 by using RT-PCR to amplify the highly conserved region of the 5′UTR. Subsequently, the positive samples were further characterized and the phylogenetic tree of the structural genes (VP3/VP1) was constructed. Moreover, the recombination event of HPeV has also been detected upon combined analysis of the phylogenetic trees of both structural and non-structural genes.

Result

Study group and epidemiology of HPeV

A total of 3716 samples were sent for influenza virus (nasopharyngeal swab) and Rota virus (stool specimens) detection (1868 females, 1833 males, and 15 with incomplete data as to their gender). All samples had been collected during the period from 2009 to 2011. Of those, 2957 NP were from 2822 individual patients whose age ranged 1 day to 90 years. All NP swabs were taken from patients who presented with influenza like-illness in the out-patient departments of two different rural hospitals. An additional 759 fecal samples were taken from 715 different individuals aged between 1 day and 24 years, who presented with acute diarrhea and sent for Rota virus detection.

Routine screening for influenza and Rota virus was accomplished by the in-house developed multiplex one-step real-time RT-PCR using TaqMan probes (Suwannakarn et al., 2008) and one-step RT-PCR (Theamboonlers et al., 2002), respectively. Glyceraldehyde 3-phosphate dehydrogenase (GADPH), the human housekeeping gene, served as an indicator for successful RNA extraction and subsequently, as an RT reaction control. This gene could be amplified in all samples (data not shown). Screening NP samples for influenza virus showed 25(0.9%) samples positive for influenza B virus, 343 samples (11.6%) positive for influenza A virus 2009 pandemic strain subtype H1, 115 samples (3.9%) were positive for influenza A virus subtype H3, and 32 samples were positive for influenza A virus whose subtype could not be classified (1.1%). Rota virus was detected in 348 fecal samples (45.9%).

Screening for HPeV was performed by using a very sensitive nested RT-PCR with the primer pair sequences retrieved from the highly conserved region of the 5′UTR for all HPeV types (Harvala et al., 2008). HPeV was thus detected in 58 out of altogether 3716 samples (1.6%) of which 46 samples were fecal samples (6.1% of fecal samples) and 12 samples were NP swab samples (0.4% of NP swab samples). The details of these 58 samples are shown in Table 1. The rate of HPeV detection did not differ between males and females. Moreover, simultaneous infection with Rota virus was detected in 16 fecal samples accounting for 34.8% of the positive stools (Table 1). One NP swab sample showed simultaneous infection with influenza A virus subtype H3 and HPeV (Table 1). The age of patients diagnosed with HPeV infection in this study ranged from 2 months to 49 years (Table 1.) however, infection was most prevalent among infants between 3 months and 1 year of age (Fig. 1A).

Typing of HPeV

For typing, all samples positive for the 5′UTR of HPeV were subjected to partial amplification of VP3/VP1 by using a highly sensitive one-step nested RT-PCR method which had proven successful for typing as described previously (Calvert et al., 2010). All 58 samples positive for the 5′UTR were subsequently subjected to direct sequencing. The phylogenetic tree shown in Fig. 2A demonstrates that the strains detected could be identified as HPeV 1A in three samples and as HPeV1B in 27 samples. Of those 27, one sample (CU-B628) was highly divergent from the rest of the group and clustered in its own clade (Fig. 2A). The divergence of CU-B628 amounted to 24% upon comparison with the Harris strain and to 18% when compared with the remaining HPeV1B clade. Furthermore, 5 samples could be identified as HPeV2; 1 sample as HPeV3; 2 samples as HPeV4; 2 samples as HPeV5; 15 samples as HPeV6; 1 sample as HPeV10 and 2 samples as HPeV14 (Table 1). All strains investigated in this study showed the majority clustered with the previously published sequences from Thailand (Fig. 2A).

The virus types identified from fecal samples were highly diverse and included HPeV1A, HPeV1B, HPeV2, HPeV3, HPeV4, HPeV5, HPeV6, and HPeV14 (Table 1 and Fig. 1B). In contrast, virus genotypes isolated from NP swab samples showed less diversity. The five genotypes identified were HPeV1A, HPeV1B, HPeV4, HPeV6 and HPeV10 (Table 1 and Fig. 1C).

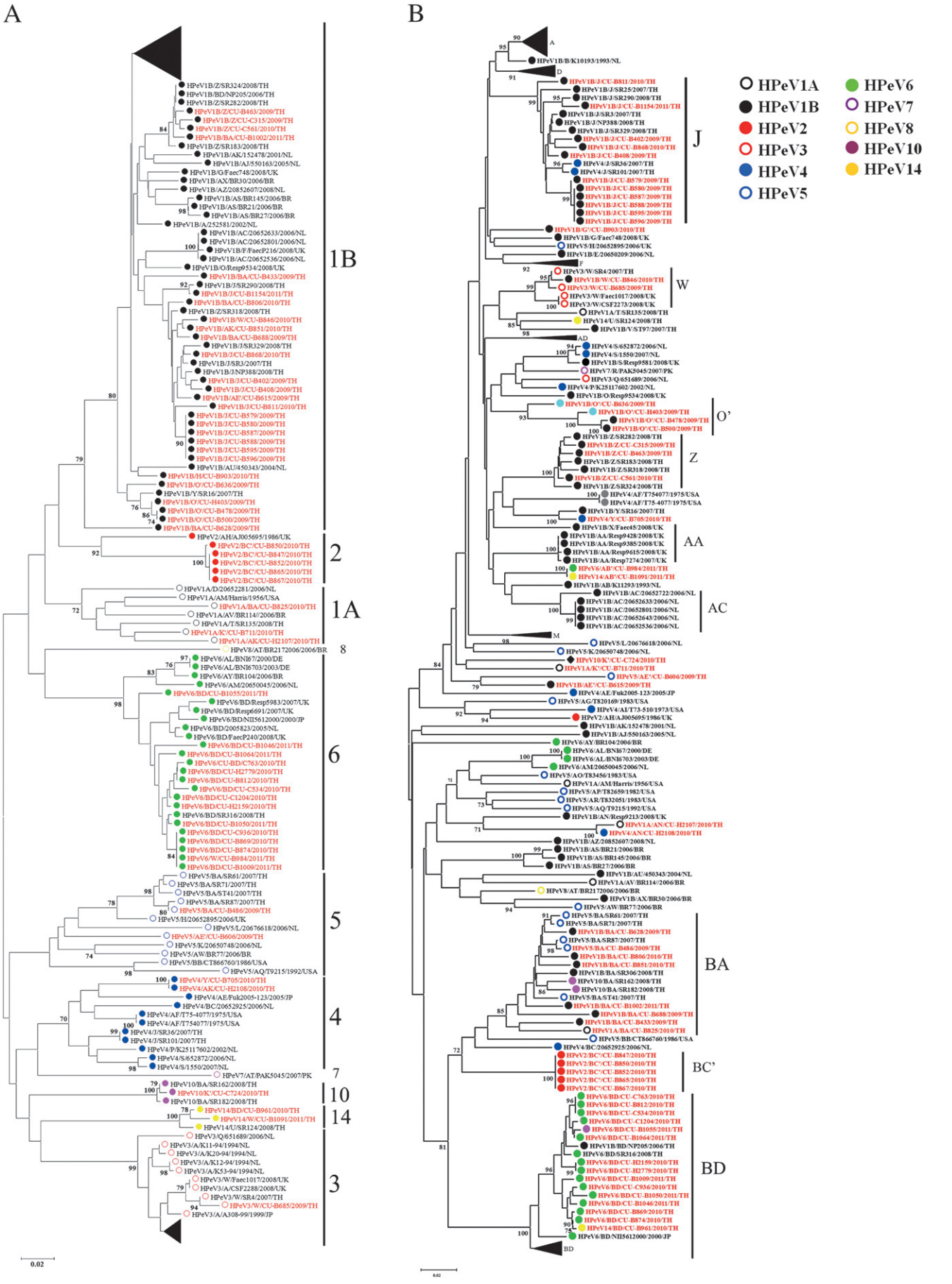
In this study, the majority of patients infected with HPeV were under 2 years of age. The most predominant genotype identified from this study, HPeV1B, was detected in patients below the age of 2 years except for one sample obtained from a 16-year-old girl (Table 1 and Fig. 1A). In contrast, HPeV6 was detected in a slightly higher age group when compared with HPeV1B. However, this difference between age groups was not statistically significant (p value = 0.302 with α level = 0.05). HPeV6 was predominantly detected in children between 7 months and 5 years of age (Table 1 and Fig. 1A). Interestingly, one fecal sample collected from a 49-year-old patient was positive for HPeV6 (Table 1 and Fig. 1A). The new types, HPeV10 and HPeV14, were identified in one NP swab sample taken from a 7-year-old girl and two fecal samples taken from two boys aged 6 months and 8 months, respectively (Fig. 1A and Table 1). HPeV1A and HPeV2 could be identified in two patient age groups, which were 3–6 months and 1–2 years (Fig. 1A). HPeV3, HPeV4 and HPeV5, had been isolated from patients of 1–2 years, 3–6 months, and 7–12 months of age, respectively (Fig. 1A and Table 1).

HPeV monthly distribution in fecal samples and NP swab is shown in Fig. 1B and C, respectively. HPeV1A could be isolated in February and May 2010 from fecal samples and July 2010 from NP swab samples (Fig. 1B and C). HPeV1B distribution in fecal samples does not seem restricted to a specific timeframe because it could be detected throughout the study period (Fig. 1B). On the other hand, in NP swab samples, HPeV1B could be detected during the first half of the study period and was then substituted by HPeV6 (Fig. 1C). HPeV2 could only be detected in stool and for a short time during the study period (July–August 2010). One strain identified as HPeV3 was isolated from a fecal sample collected in December 2009 (Fig. 1B). HPeV4 was detected in a fecal sample collected in January 2010 and an NP swab sample collected in July 2010 (Fig. 1B and C). Interestingly, HPeV5 was only detected at the very beginning of the study (January and March 2009) and subsequently disappeared (Fig. 1B). The new types, HPeV10 and HPeV14, were detected in March 2010 (Fig. 1C) and February 2011(Fig. 1B), respectively.

VP1 divergence

The substitution rate in the partial VP3/VP1 gene of all HPeV genotypes was 3.02×10^{-3} nucleotides per site per year (with 2.29×10^{-3}–3.83×10^{-3} as highest posterior density; HPD, interval). Information on virus sequences of genotypes 1A, 2, 4, 5, and 7–16 is limited. Therefore, the nucleotide change rate could only be

Fig. 2. Phylogenetic tree of partial VP3/VP1 (A) and 3Dpol gene (B). Each branch was labeled as genotype/3D clade/strain name/year of collection/origin country. The different colors represent the different HPeV genotypes which were classified based on the cluster on the phylogenetic tree of the VP3/VP1 gene (A). The samples from this study are indicated as red letters on each branch of the tree. Only the bootstrap with over 70 were shown in phylogenetic tree and the large clades that the study samples did not appeared were collapsed and shown as the black triangle in the tree.


A
HPeV1B/Z/SR324/2008/TH
HPeV1B/BD/NP205/2006/TH
HPeV1B/Z/SR282/2008/TH
HPeV1B/Z/CU-B463/2009/TH
HPeV1B/Z/CU-C315/2009/TH
HPeV1B/Z/CU-C561/2010/TH
HPeV1B/BA/CU-B1002/2011/TH
HPeV1B/Z/SR183/2008/TH
HPeV1B/AK/152478/2001/NL
HPeV1B/AJ/550163/2005/NL
HPeV1B/G/Faec748/2008/UK
HPeV1B/AX/BR30/2006/BR
HPeV1B/AZ/20852607/2008/NL
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HPeV4/S/1550/2007/NL
HPeV1B/S/Resp9581/2008/UK
HPeV7/R/PAK5045/2007/PK
HPeV3/Q/651689/2006/NL
HPeV4/P/K25117602/2002/NL
HPeV1B/O/Resp9534/2008/UK
HPeV1B/O'/CU-B636/2009/TH
HPeV1B/O'/CU-H403/2009/TH
HPeV1B/O'/CU-B478/2009/TH
HPeV1B/O'/CU-B500/2009/TH
HPeV1B/Z/SR282/2008/TH
HPeV1B/Z/CU-C315/2009/TH
HPeV1B/Z/CU-B463/2009/TH
HPeV1B/Z/SR183/2008/TH
HPeV1B/Z/SR318/2008/TH
HPeV1B/Z/CU-C561/2010/TH
HPeV1B/Z/SR324/2008/TH
HPeV4/AF/T754077/1975/USA
HPeV4/AF/T75-4077/1975/USA
HPeV1B/Y/SR16/2007/TH
HPeV4/Y/CU-B705/2010/TH
HPeV1B/X/Faec45/2008/UK
HPeV1B/AA/Resp9428/2008/UK
HPeV1B/AA/Resp9385/2008/UK
HPeV1B/AA/Resp9615/2008/UK
HPeV1B/AA/Resp7274/2007/UK
HPeV6/AB'/CU-B984/2011/TH
HPeV14/AB'/CU-B1091/2011/TH
HPeV1B/AB/K11293/1993/NL
HPeV1B/AC/20652722/2006/NL
HPeV1B/AC/20652633/2006/NL
HPeV1B/AC/20652801/2006/NL
HPeV1B/AC/20652643/2006/NL
HPeV1B/AC/20652536/2006/NL
HPeV5/L/20676618/2006/NL
HPeV5/K/20650748/2006/NL
HPeV10/K'/CU-C724/2010/TH
HPeV1A/K'/CU-B711/2010/TH
HPeV5/AE'/CU-B606/2009/TH
HPeV1B/AE'/CU-B615/2009/TH
HPeV4/AE/Fuk2005-123/2005/JP
HPeV5/AG/T820169/1983/USA
HPeV4/A/A1/T73-510/1973/USA
HPeV2/AH/AJ005695/1986/UK
HPeV1B/AK/152478/2001/NL
HPeV1B/AJ/550163/2005/NL
HPeV6/AY/BR104/2006/BR
HPeV6/AL/BNI67/2000/DE
HPeV6/AL/BNI6703/2003/DE
HPeV6/AM/20650045/2006/NL
HPeV5/AO/T83456/1983/USA
HPeV1A/AM/Harris/1956/USA
HPeV5/AP/T82659/1982/USA
HPeV5/AR/T832051/1983/USA
HPeV5/AQ/T9215/1992/USA
HPeV1B/AN/Resp9213/2008/UK
HPeV1A/AN/CU-H2107/2010/TH
HPeV4/AN/CU-H2108/2010/TH
HPeV1B/AZ/20852607/2008/NL
HPeV1B/AS/BR21/2006/BR
HPeV1B/AS/BR145/2006/BR
HPeV1B/AS/BR27/2006/BR
HPeV1B/AU/450343/2004/NL
HPeV1A/AV/BR114//2006/BR
HPeV8/AT/BR2172006/2006/BR
HPeV1B/AX/BR30/2006/BR
HPeV5/AW/BR77/2006/BR
HPeV5/BA/SR61/2007/TH
HPeV5/BA/SR71/2007/TH
HPeV1B/BA/CU-B628/2009/TH
HPeV5/BA/SR87/2007/TH
HPeV5/BA/CU-B486/2009/TH
HPeV1B/BA/CU-B806/2010/TH
HPeV1B/BA/CU-B851/2010/TH
HPeV1B/BA/SR306/2008/TH
HPeV10/BA/SR162/2008/TH
HPeV10/BA/SR182/2008/TH
HPeV5/BA/ST41/2007/TH
HPeV1B/BA/CU-B1002/2011/TH
HPeV1B/BA/CU-B688/2009/TH
HPeV1B/BA/CU-B433/2009/TH
HPeV1A/BA/CU-B825/2010/TH
HPeV5/BB/CT866760/1986/USA
HPeV4/BC/20652925/2006/NL
HPeV2/BC'/CU-B847/2010/TH
HPeV2/BC'/CU-B850/2010/TH
HPeV2/BC'/CU-B852/2010/TH
HPeV2/BC'/CU-B865/2010/TH
HPeV2/BC'/CU-B867/2010/TH
HPeV6/BD/CU-C763/2010/TH
HPeV6/BD/CU-B812/2010/TH
HPeV6/BD/CU-C534/2010/TH
HPeV6/BD/CU-C1204/2010/TH
HPeV6/BD/CU-B1055/2011/TH
HPeV6/BD/CU-B1064/2011/TH
HPeV1B/BD/NP205/2006/TH
HPeV6/BD/SR316/2008/TH
HPeV6/BD/CU-H2159/2010/TH
HPeV6/BD/CU-H2779/2010/TH
HPeV6/BD/CU-B1009/2011/TH
HPeV6/BD/CU-C936/2010/TH
HPeV6/BD/CU-B1050/2011/TH
HPeV6/BD/CU-B1046/2011/TH
HPeV6/BD/CU-B869/2010/TH
HPeV6/BD/CU-B874/2010/TH
HPeV14/BD/CU-B961/2010/TH
HPeV6/BD/NII5612000/2000/JP
J
W
AD
O'
Z
AA
AC
M
BA
BC'
BD
HPeV1A
HPeV1B
HPeV2
HPeV3
HPeV4
HPeV5
HPeV6
HPeV7
HPeV8
HPeV10
HPeV14
0.02

investigated in the three genotypes of HPeV1B, HPeV3 and HPeV6. The result shown in Table 2 indicates that these three genotypes were in a neutral selection condition based on the ratio of non-synonymous change per site to synonymous change per site (d_N/d_S). In this study, the substitution rate of HPeV1B amounted to 9.11×10^{-3} substitutions per site per year exceeding by more than 3 times that of HPeV3 and by nearly 2 times that of HPeV6 with 3.03×10^{-3} and 5.10×10^{-3}, respectively (Table 2).

Recombination event of HPeV

Together with the structural gene, the non-structural 3D gene at the 3′end of the virus genome responsible for RdRp activity was used to investigate the recombination event (Calvert et al., 2010). The specific 3D gene primer sets were incorporated into a one-step nested RT-PCR method described elsewhere (Calvert et al., 2010). All HPeV positive samples with successful amplification of the 5′UTR and VP3/VP1 were also subjected to this 3D amplification and direct sequencing. The phylogenetic tree of the 3D gene is shown in Fig. 2B and the specific clade assigned for each HPeV positive sample is shown in Table 1. Interestingly, the high divergence rate ranged from 6.2% to 16.1% when compared with the nearest clade on the phylogenetic tree of 3Dpol gene was shown in sixteen strains of viruses identified in this study (Supplement Table S2). As defined in a previous study, the 5.5% divergence from each other was shown as the cut off for a different 3Dpol gene clade assignment (Calvert et al., 2010). Therefore, based on this demarcation, we could identify 6 additional 3Dpol clades. The samples identified in newly assigned clades of 3Dpol are shown in Table 1 and Fig. 2B. For recombination analysis, the discordant grouping based on the 3Dpol gene of the genotype characterized by the VP3/VP1 gene phylogeny is indicative of recombination (Calvert et al., 2010). Therefore, in this study recombination was detected in 16 samples including 3 strains of HPeV1A (CU-B711, CU-B825 and CU-H2107); 7 of HPeV1B (CU-B433, CU-B615, CU-B688, CU-B806, CU-B846, CU-B851, and CU-B1002); 2 of HPeV4 (CU-B705 and CU-H2018); 1 of HPeV5 (CU-B606); 1 of HPeV6 (CU-B984) and 2 of HPeV14 (CU-B961 and CU-B1091). The most frequently recombination event was shown in HPeV1B in which could be classified into 3 clades of 3Dpol including BA, AE′ and W (Table 1). The 3Dpol clades that were assigned to the remaining recombination strains are shown in Table 1. The recombination detection rate is 27.6%, this may be underlining the detection rates which based on samples and children.

Discussion

Molecular epidemiology

Until recently, based on their genomic sequences, 16 genotypes of virus have been discovered and subsequent studies have shown that the different genotypes of virus cloud provide the different diseases, for example, HPeV1 is associated with diseases of the GI tract and HPeV3 is closely related with sepsis and encephalitis in neonates (Abed and Boivin, 2006; Boivin et al., 2005; Harvala et al., 2009). Epidemiology of HPeV is required to further our understanding of disease causation and possibly, geographic distribution of the virus.

Recently, molecular techniques were used for detection and genotype classification. For screening, nested RT-PCR with a primer pair specific for the conserved region of the 5′UTR was applied. The result showed that 1.6% of samples (58 of 3716 samples) were positive for HPeV with more than one third originating from the GI tract (feces = 46; 6.1%, NP swab = 12; 0.4%) (Table 1). The percentage of positive samples in this study was lower than in the previous report, 1.2% of respiratory samples (Harvala et al., 2008) and 16.3% of fecal samples (Benschop et al., 2008b). However, the molecular epidemiology data presented in this study was based on the number of samples rather than the number of patients which might account for the lower detection rate. The difference in virus detection rate may well be due to the specific year of sample collection and geographic location. Interestingly, the study of Pham et al. conducted in 2010 which included stool samples negative for human rotavirus from the northern part of Thailand showed 14.6% positive for HPeV. In another way, if the positive samples of human rotavirus were excluded in this study, the percentage of HPeV positive (11.2%) stool samples has shown a similar range as the study of Pham et al. in 2010.

As reported by previous studies, the majority of HPeV positive samples originated from patients below the age of 2 years with the highest rate shown among 3 month-to-1-year olds (Fig. 1A). HPeV1B is the most prevalent genotype in samples collected from children, whereas HPeV6 seems to infect older patients (Fig. 1A). However, a larger number of samples positive for other genotypes should be considered for future analysis to further our understanding of age distribution for this virus. Interestingly, in this study, HPeV was detected in three patients above 10 years of age. It is unusual to detect HPeV in adults but a few studies have reported HPeV detection in over 10-year-olds (Abed and Boivin, 2006; Figueroa et al., 1989; Li et al., 2009; Tapia et al., 2008). Therefore, this provides additional evidence of HPeV infection in patients above the age of 10 years. Unfortunately, since the samples included in this study were anonymous, further information on these three patients as for example, any underlying disease could not be obtained. (Table 1).

The cycle of HPeV infection, occurring twice per year, in summer (April to June) and winter (December to February), has been shown in this study (Fig. 1B and C). The same seasonal occurrence of HPeV infection has been reported by Harvala et al. (2008) in Scotland. In our study had identified the only one sample that defined as HPeV3 during the winter season. As same as previously reported, HPeV3 could be identified in the winter season (Abed and Boivin, 2006; Harvala et al., 2009). However, the HPeV3 was detected during the summer season had also been reported (Benschop et al., 2006b). In this study, two types of HPeV, HPeV1 and HPeV6, were predominant in respiratory samples. This is in agreement with the data presented by Havala et al. in 2008 (Fig. 1C). In contrast, in fecal samples, only HPeV1B was predominant and spread throughout the study period (Fig. 1B). In addition, HPeV2 was frequently identified in our study, while this type of virus has not been detected in any other recent study (Benschop et al., 2008b; Harvala et al., 2008).Additional epidemiology studies might provide further understanding of unusual or new types of HPeV.

Typing, recombination and evolution of HPeV

Based on the phylogeny of the VP3/VP1 gene, a profound diversity of HPeV genotypes was found in this study with the eight genotypes 1A, 1B, 2, 3, 4, 5, 6 and two newly recently identified genotypes, 10 and 14, classified (Fig. 2A). Genotype 1B was predominant and upon comparison between the 6 different lineages as showed multiple variations in Fig. 2A. Interestingly, CU-B628 was separate in its own clade on the phylogenic tree of the VP3/VP1 gene and the divergence was equal to 24% when compared with the Harris strain. Since the divergence for new genotype assignment has to exceed 25%, unfortunately, this CU-B628 had still been assigned to genotype 1B. Although these characteristics are insufficient to assign a new genotype to this strain, its variety should be addressed. The viruses identified from this study

Table 2
Estimate of sequence rate change of HPeV1B, 3, and 6.

HPeV type	Distance*	d_N/d_S*	Substitution rate ($\times10^{-3}$)	MRCA
1B	0.1000	0.0099	9.11 (5.14–13.36)	1985 (1978–1991)
3	0.0346	0.0181	3.03 (1.57–4.49)	1990 (1985–1995)
6	0.0607	0.0334	5.10 (2.32–8.24)	1994 (1987–1999)

* Calculated by using maximum composite likelihood as the substitution model.

were grouped together on the phylogenetic tree of VP3/VP1 gene and also clustered with viruses previously reported from Thailand (Fig. 2A). This indicates a geographically specific common determinant that appears to be found on this VP3/VP1 phylogenetic tree of HPeV. Moreover, difference of the HPeV genotype could be detected in children below 2 years of age.

Altogether, the genotypes have not evolved quite as rapidly as the substitution rate calculated by BEAST suggested (3.02×10^{-3} substitutions per site per year) which was slightly higher than that previously proposed for the VP1 gene (Faria et al., 2009). However, along with phylogenetic analysis, a vast difference in substitution rate was shown among different genotypes of virus (Table 2). Incorporation of much more samples used for the calculation may be the reason why the evolution rate in this study appeared slightly higher than that previously estimated (Calvert et al., 2010). Similarly, the d_N/d_S calculated from this study also were slightly higher than previously mentioned by Calvert et al. (2010). In contrast, the MRCA shown in our study (Table 2.) did not show any difference to the previous report (Calvert et al., 2010). Further studies on larger sample sizes will provide further understanding of virus evolution.

Interestingly, as defined in the previous study, more than 5.5% divergence was shown as the cut off for a different 3Dpol gene clade assignment (Calvert et al., 2010). Hence 6 additional new 3Dpol clades could be defined based on our study samples (Table 1.).

Recombination of HPeV had previously been addressed with the recombination breaking points established at the junction between 5′UTR and P1 and the P1 and P2 polyprotein (Benschop et al., 2008a, 2010; Drexler et al., 2011; Williams et al., 2009; Zoll et al., 2009). Despite the phylogenetic analysis comparison between structural and non-structural genes such as the 3Dpol gene was successfully used to investigate recombination (Calvert et al., 2010). The discordance cluster in the 3Dpol gene when compared with the VP3/VP1 gene phylogenetic tree proves that recombination in the virus genome has occurred (Calvert et al., 2010). Most of the samples from our study were classified as the same clade for 3Dpol phylogenetic tree as the reported in Thai samples that reported by Calvert et al. (2010) (Table 1). Sixteen samples (27.6%) showed discordance between two genetic regions on the phylogenetic tree (Fig. 2) serving as evidence for recombination from this study. HPeV1B was the predominant genotype with a recombination frequency of 27%. This study was in agreement with previous research emphasizing the high recombination frequency of HPeV type 1B (Calvert et al., 2010). In contrast, HPeV6 exhibited less recombination frequency (6%) with only one sample identified (CU-B984, Fig. 2.). Other genotypes including HPeV1A, HPeV4, HPeV5, and HPeV14 had also undergone recombination (Fig. 2.), however, the frequency of recombination had not been done due to the small identification numbers from the study. HPeV3 isolated from this study clustered in the correlated phylogenetic tree clade with both VP3/VP1 and 3Dpol genes when compared to the previous report (Fig. 2.) (Calvert et al., 2010). Therefore, the recombination might not have been detectable in this study. The underlying factors or mechanisms for the recombination event are still unclear and should be addressed by further investigation.

In conclusion, although HPeV has been discovered a long time ago, its biology, epidemiology, evolution and pathogenicity are still unclear. Therefore, ongoing molecular research will further our understanding of this virus which can also be applied to other related viruses.

Method

Clinical samples

The nasopharyngeal (NP) swabs were collected from patients presenting with influenza-like illness. A total of 2957 of NP swab samples were collected between July 14, 2009 and March 9, 2011. All samples were sent from local hospitals including; 1703 samples from Chumphae hospital, Chumphae district, Khonkaen province, 1254 samples from Thungsong hospital, Thungsong district, Nakorn Sri Thammarat province. Samples were kept in transport media which contain phosphate-buffered saline (PBS) with antibiotics (2×10^6 U/l of penicillin G and 200 mg/l of streptomycin). All were kept on ice then sent to Center of Excellence in Clinical Virology, Department of Pediatrics, Faculty of Medicine, Chulalongkorn University for the routine screening. Samples were processed immediately upon arrival, the remaining were kept at −70 °C for further use.

Fecal samples were sent to our center for Human Rota virus routine screening. A total of 715 fecal samples had been collected in the 2-year period of January 7, 2009 to April 18, 2011. Of those, 12 samples were sent from Umphang hospital, Umphang district, Tak province; 597 samples from Chumphae hospital, Chumphae district, Khonkaen province and 150 samples from King Chulalongkorn Memorial hospital, Bangkok. Upon arrival, fecal samples were diluted 1:10 with PBS, thoroughly mixed on a vortex and centrifuged at 8000 rpm. Supernatants were collected and stored at −70 °C until further use.

RNA extraction and HPeV detection

All samples were subjected to RNA extraction by conventional GTC-phenol-chloroform method performed on 200 µl each of clinical samples. The extracted RNA was stored at −70 °C until used.

All extracted RNA was subjected to reverse transcription into cDNA using the ImProm-II™ Reverse Transcription system (Promega, Madison, WI). Reverse transcription (RT) was performed by following the manufacturer's specifications and a random hexamer primer was used for the RT step. cDNA was stored at −20 °C until used.

Nested-PCR for HPeV detection was modified from a previous study (Harvala et al., 2010). The PerfectTaq Plus MasterMix (5 PRIME, Darmstadt, Germany) was used as the amplification mixture. The predicted positive result was shown as a single band of 243 bp when visualized under UV light after 2% agarose gel electrophoresis and staining with ethidium bromine.

Partial VP3/VP1 and 3Dpol gene amplification

For HPeV characterization, the samples positive for the 5′UTR were subjected to nested PCR to amplify the VP3/VP1 junction for HPeV typing as previously described (Harvala et al., 2010). The expected PCR product was 304 bp when virtualized under UV light after 2% agarose gel electrophoresis and staining with ethidium bromine.

The partial 3Dpol gene was amplified by nested PCR modified from a previous report (Calvert et al., 2010). The expected second round PCR product of 700 bp was virtualized under UV light after 2% agarose gel electrophoresis and staining with ethidium bromine.

All VP3/VP1 and 3Dpol positive amplicons were purified by using Agarose Gelextract Mini Kit (5 PRIME, Darmstadt, Germany). Direct sequencing was performed under 1st BASE DNA Sequencing Services (1st BASE Laboratories, Malaysia). Sequencing results were annotated, aligned, and managed by using combination of software including Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi), Simmonics version 1.7 (www.virus-evolution.org), Chromas Lite (http://www.technelysium.com.au/chromas_lite.html), and BioEdit version 7.0.4.1(Hall, 1999).

Molecular and evolution analysis

All study sequences had been submitted to GenBank data base. The accession number that included in this study was show in Supplement Table (S1). Phylogenetic trees were constructed by using MEGA 4 software (Tamura et al., 2007) in which neighbor-joining method with 1000 replication for bootstrapping was implemented. Trees contracted under maximum-composite-likelihood (MCL) as

distances model and using pairwise deletion as gap/missing data processing. The nucleotide substitution rate and most recently common ancestor (MRCA) were achieved by using Bayesian Evolutionary Analysis by Sampling Trees (BEAST) software version 1.6 (Drummond and Rambaut, 2007). The relaxed clock-uncorrelated exponential with 10 million chains was run under GTR with gamma distribution substitution model. The data from BEAST were analyzed by TACER program (http://beast.bio.ed.ac.uk/Tacer).

Ethical consideration

All samples that were included in this study were sent for routine diagnosis in our center. The personal data such as name and HN did not appear in any part of the document in this study. All samples were taken with permission from the Director of Chulalongkorn King Memorial hospital. Moreover, the study was conducted after the approval of the Ethics Committee of the Faculty of Medicines, Chulalongkorn University (IRB No.086/53).

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